Institut für Veterinärpathologie
Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. Andreas Pospischil

Arbeit unter Leitung von
Dr. Phil.II Enrico Brugnera und Dr. Titus Sydler

„Porcine circovirus 2 genotype groups and subgenotypes coreplication a major contributing factor to disease riddle?“

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Siegfried Khaiseb

Tierarzt
von Outjo, Republik Namibia

genehmigt auf Antrag von

Prof. Dr. Andreas Pospischil, Referent
Prof. Dr. Mathias Ackermann, Korreferent

Zürich 2011

CONTENTS

SUMMARY

A *Circoviridae* family member, porcine circovirus type 2 (PCV2), is associated with postweaning multisystemic wasting syndrome (PMWS), a recent emerging disease worldwide. PCV2 is also found in clinical asymptomatic animals. This paradoxical finding makes the syndrome etiology challenging. We developed new assays to study PCV2 with links to syndrome etiology. For analysis we used PCV2 infected tissue from subclinical infected and diseased piglets. We compared antigen and PCV2 DNA derived signals in tissue localization and intensity. We designed oligonucleotides to the signature motif of the PCV2 capsid open reading frame to discriminate experimentally between PCV2 genotype groups by PCR, in situ hybridization (ISH) and fluorescence in situ hybridization (FISH). With genotypic PCR and ISH, we found PCV2 infected animals to carry both PCV2a and PCV2b genotype groups. We observed genotype single cell infection and superinfections by both genotype groups. By FISH, we discriminated replicative DNA isoform from total PCV2 DNA isoforms. We extended this method to inquire genotype group specific replication. Single genotype group replication was not observed. These findings suggest PCV2 genotype relaxed replication regulation requirements and, may even point to genotype group replication cooperativity *in vivo*. These observations explain the readily seen PCV2 DNA recombination and the high overall PCV2 genome plasticity. We propose a novel mechanism for syndrome ethiology.

INTRODUCTION

Members of the *Circoviridae* family and, through interspecies recombination, related viruses cause diseases in vertebrates and plants [1,2]. Viruses' interspecies recombination was linked to disease outbreak [3]. *Circoviridae* members PCV2 and its apathogenic PCV1 relative are the smallest autonomous replicating viruses known in eukaryotic cells. PCV2 and PCV1 genomic sequences elicit less than 80% sequence homology. They are non-enveloped viruses that posses a closed circular, single-stranded DNA (ssDNA) genome [4]. Upon infection, the ssDNA genome is converted into a double-stranded (ds) intermediate, the replicative virus DNA isoform [5], which serves as template for rolling-circle synthesis of the viral ssDNA [6]. PCV replication occurs by the so-called melting-pot rolling-circle replication mechanism [7]. Of note, per one ds PCV2 DNA molecule, one new ss PCV2 DNA is formed [7].

The small genome of PCV2 contains at least three open reading frames (ORFs) with known function: ORF1 codes for two replicase proteins, ORF2 for the structural protein (cap gene) and ORF3 for a protein implicated in cellular apoptosis that overlaps with ORF1 [8,9]. The PCV2 genome is ambisense i.e. the encapsidated viral DNA strand which serves as a template for transcription of the capsid protein gene (ORF2), while the complementary DNA strand of the replicase functions as transcription template for the replicase gene (ORF1) [9].

PCV2 has recently emerged in pigs potentially as the result of a cross-species jump from birds into domestic pigs [10], most likely through the wild boar intermediary host in less than a hundred years [10]. PCV2 adaptation to new hosts is truly remarkable and adaption is not restricted to or ends with pigs as other research groups [11,12] and we (unpublished data) found PCV2 in bovine. Also, other studies describe PCV2 presence in mouse and human [13,14]. One definite contributing characteristic is the viral genomic plasticity at the mutational rate almost as high as it is described for RNA viruses [10].

Indeed, PCV2 seems the primary etiological agent for PMWS, nevertheless infections are more prevalent than disease and, in animal infection experiments severe clinical signs are rarely manifested. In PCV2 infected animals a transient lymphopenia is often observed and, in neonates, a general depression of the immune system (Darwich et al 2004; McCullough et al 2007). PMWS is an excellent example of a *Circoviridae* caused disease. It is comparably a recent disease as the first cases occurred early 1990s in Western Canada [15]. Although PMWS clinical symptoms are well described with wasting and enlarged lymph nodes [16] and microscopic lesions in lymphoid organs by lymphocyte depletion and histocytic infiltrations, the syndrome appears multifactorial triggered with a poor defined disease etiology [17]. Hence, researchers suggested cofactors as essential for PMWS development: these cofactors include other bacterial or viral infections, over-stimulation or immune-suppression, a general genetic shift in the pig population or pig management changes [18]. However, none of these cofactors were evident for the Swiss epizooty [19].

Phylogenetical studies divided PCV2 viruses into two major genotype groups namely PCV2a and PCV2b [20]. A simple distinction between PCV2a and PCV2b genotypes was achieved by comparing a distinct stretch of amino acids in the viral capsid protein, the so-called signature motif [21]. By this criteria also, PCV2c [22] a third genotype group was included within PCV2b genotype group. A genetic shift from PCV2a to new

emerging PCV2b genotype group was detected in Canadian and USA PMWS outbreaks [21,23]. In analogy, we also found a dominance of PCV2b genotype group during the epizooty however PCV2b genotype group members were also found before the epizooty with dominant PCV2a genotype group occurrence [19]. Additionally we found, beside the PCV2b genotype group dominance, a subgenotype shift [19] in correlation to the PMWS epizooty. Interestingly, various research groups found further evidence for different virulence of PCV2 variants *in vitro* and in animal infection models [24-26]. Although substantial efforts were undertaken neither genotype group nor subgenotype was directly correlated with PMWS disease [27,28].

To complicate the mechanism of disease onset it was seen that infected animals carry multiple PCV2 genotype groups and subgenotypes with a possible link to PMWS occurrence [29,30]. Additionally, several studies presented sequence evidence for PCV2 genotype group and subgenotype recombination [30-32]. Recombination events were observed by sequence analysis in both major PCV2 ORFs [28,33]. The contribution of recombination to PCV2 genomic evolution may be the critical factor to initiate PMWS in an animal's immune system already weakened by a PCV2 caused lymphopenia. As different studies showed, recombination for other viruses may be linked to zoonoses and severe disease outbreaks [1,3].

In order for virus recombination to take place, at least two different non-competitive viruses' DNA need to intracellularly co-replicate. By PCR, ISH and FISH technologies we studied the presence of major genotype groups in piglets at the organs and cellular levels. In this work, we consistently noticed the presence of both major PCV2 genotype groups in PCV2 pre-epizootic and post-epizootic infected piglets indiscriminately of virus concentration within the animal. We confirmed our previous observations that PCV2a genotype group dominated pre-epizootic and, PCV2b, more specifically PCV2b-CH, during the epizooty. Interestingly, we found superinfection and co-replication of both PCV2 major genotype groups in the same cell. Surprisingly, however, was the occurrence of PCV2 *in vivo* replication exclusively to cells with both genotype group members although virus genotype group single cell infections were also seen.

RESULTS

Validation of PCV2 genotype group specific PCR amplification and PCV2 genotype group distribution

We surmised about double genotype group infections and correlation to disease. Therefore, we first optimized PCV2a and PCV2b template specific PCR methods by construction of respective PCV2 genotype sequence containing vectors. We titrated individual constructs and optimized PCR conditions to detect as low as 10 templates per reaction (Figure 1). Genotype group specific oligonucleotides were designed within the signature motif to specifically distinguish all known PCV2a from PCV2b subgenotypes. These oligonucleotides are different in 2bp at 3' prime end that prevents cross-reaction amplification products even in template concentrations as high as 10^7- 10^8 templates per reaction (Figure 1). These template concentrations compare to the highest we found from DNA preparations of PMWS diseased animals.

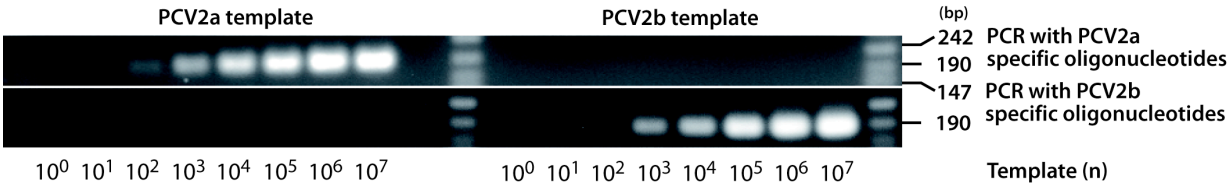

Figure 1. Genotypic PCR specificity and sensitivity for PCV2a genotype group and PCV2b genotype group templates. Two panels show each separate agarose gel electrophoreses of amplification products from PCV2a or PCV2b genotype group specific PCR reactions. From left to right panels are further divided by DNA ladder into PCR reactions against plasmids containing PCV2a or PCV2b sequence. At the lower bottom, numbers indicate serial template dilutions (10^0-10^7) from corresponding plasmids. On the right side of the panels is the molecular weight of single band of the DNA ladder in base pairs (bp) indicated.

From tissue block collection we selected 48 cases by two criteria (Table): we analysed cases from the time period before and during the Swiss PMWS epizooty; with no, low or medium to high antigen concentration identified by immune-histochemically immunohistochemistry (IHC) (Table). These tissue blocks were further re-evaluated by three different PCR methods. A PCV2 specific oligonucleotide set from our recent study allowed us to amplify the dominant PCV2 subgenotype infection indiscriminately of genotype group affiliation for each case and tissue. PCV1 was not detected by this PCV2 specific PCR amplification. Of note, data indicate that the optimized PCR method is more sensitive than IHC (Table). From real-time PCR data we estimated that a PCR amplification signal was at least three logarithms virus genome more sensitive than PCV2 antigen-antibody signals (unpublished data). By amplificate sequencing, we found PCV2b genotype group infections predominately occurring during the epizooty

reminiscent to our previous findings: 27% before and 97% during the Swiss epizooty were of the PCV2b genotype group. In fact, all 30 PCV2b group infections from the epizooty harboured PCV2b-CH subgenotype. Separately, for four randomly selected PCV2 infected cases we analysed different organs, including lung, spleen, kidney and lymph node by PCR and sequencing. Of note, the same virus variant dominantly infected all PCV2 infected organs of the same animal. By genotype group specific PCR amplification we searched for the presence of PCV2a or PCV2b genotype group infections. We found that all animals were harbouring both genotype groups including the 6 cases that were IHC negative, indiscriminately from time of case occurrence. Actually, in many PCV2b genotype group specific PCR amplificate separated by agarose gel electrophoreses appeared more intense than the PCV2a genotype group signal from the same case during the epizooty and vise versa pre-epizootic. It was puzzling to find both genotype groups in all infected tissue sections especially as no molecular controls indicated any contamination. However, it was yet to be shown whether there was a coincidental contamination.

Clinical diagnosis	Before PMWS epizooty	ISH signal intensity (n) with probe AB	Dominant PCV2 genotype (n)	ISH(PCV2a/b) with probe A + B and PCR(PCV2a/b) positive (n)
Subcl. infec.	IHC neg.	5 +	2a/3b	5
Subcl. infec.	IHC +	2 +	2a	2
PMWS	IHC ++	2 ++	2a	2
PMWS	IHC +++	6 +++	5a/1b	6
	Piglet cases	15	15	15
	During PMWS epizooty			
Subcl. infec.	IHC neg.	1 +	1a	1
Subcl. infec.	IHC +	4 +	4b	4
PMWS	IHC ++	2 ++ / 4 +	6b	6
PMWS	IHC +++	5 +++/ 9 ++/ 8 +	22b	22
	Piglet cases	33	33	33

Table. Comparison of fixed and paraffin embedded piglet tissue sections before and during epizooty by PCV2 specific IHC, ISH and PCR. This is a summary of the 48 piglet cases analysed. PMWS clinical diagnosis, IHC and Swiss epizooty were previously described [19]. ISH signal intensity comparisons were achieved with aid of oligonucleotide AB tissue section labelling. The oligonucleotide AB recognizes any PCV2 genotype group members. Additionally, the dominant genotype group (a or b) was determined with PCV2 specific PCR amplification and product sequencing [19]. PCV2 genotype specific ISH and PCR revealed that all investigated infected tissue samples contain both genotypes PCV2a and PCV2b (PCV2a/b with oligonucleotide probes A + B). The + indicates the staining strength with +++ indicating the strongest label; the staining intensity begins with negative (neg.; no cell was visibly labelled) and one + to three +++. Subclinically infected (Subcl. infec.) piglets with PCV2 infections were clinically inconspicuous. The number of investigated piglets are indicated with n.

In situ hybridization specificity and sensitivity supports PCR derived data showing presence of both genotype groups in PCV2 infected animals

We decided to further analyze these 48 cases by ISH. Its advantage over PCR is the signal-defined localization. Moreover, ISH derived signal localization was compared to well-defined antigen staining. Coincidental contamination from organ environment could be readily distinguished by ISH. For optimization of signal intensity we first titrated probes on low to high PCV2 antigen content containing tissue sections. We compared oligonucleotide hybridization reactivity to non-PCV2 infected pig tissue and other species, including cat, snake and dog organ sections. These negative controls also included sections with high concentration of hyaluronic acid, necrotic and apoptotic tissue. Even with 5-fold higher oligonucleotide concentration than what we finally applied in hybridization reaction, we did not find any unspecific reaction signals on tissue sections. We compared ISH signal localization and intensity to IHC signals. We found reminiscent to antigen tissue localization, PCV2-DNA derived signals centro-follicular and interspersed in surrounding tissue probably caused by infected immigrating macrophages (Figure 2a). Of specific note is the signal sensitivity by PCR and ISH. Both methods are superior to IHC for the detection of low PCV2 infections (Table). Apart from PCR positive IHC negative tissue sections, signal intensity from low to high PCV2 infected tissues correlates well between IHC and ISH (Table). In few high PCV2 antigen-containing tissues from PMWS cases correlation between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions was not observed. In these tissues cellular structural integrity was questionable and PCV2 DNA concentration dependent signals varied from low to high.

Template specificity of signals detected by ISH

For detailed analysis, we identified what type of nucleic acids are recognized by oligonucleotides, whether the ISH signals were the result of the oligonucleotide hybridization to DNA, RNA or both targets. We applied serial dilutions of digestions with corresponding activity to remove DNA or RNA targets. We selected consecutive spleen sections from a PMWS diseased animal with a medium to high antigen concentration. At this PCV2 load, splenic tissue ultrastructure was fully conserved. However, we noticed histological lesions with lymphocyte depletion and histiocytic inflammatory infiltration typical for PMWS. ISH derived blue staining signals were located mostly in histocytes (Figure 2), which correlated also to IHC signals (data not shown). We observed in the Dnase I as well as RNase buffer controls, increased ISH signal intensity

throughout the different experiments (Figure 2a, 2b). One hour digestion with 50 U DNase I enzyme obliterated signals completely (Figure 2c).

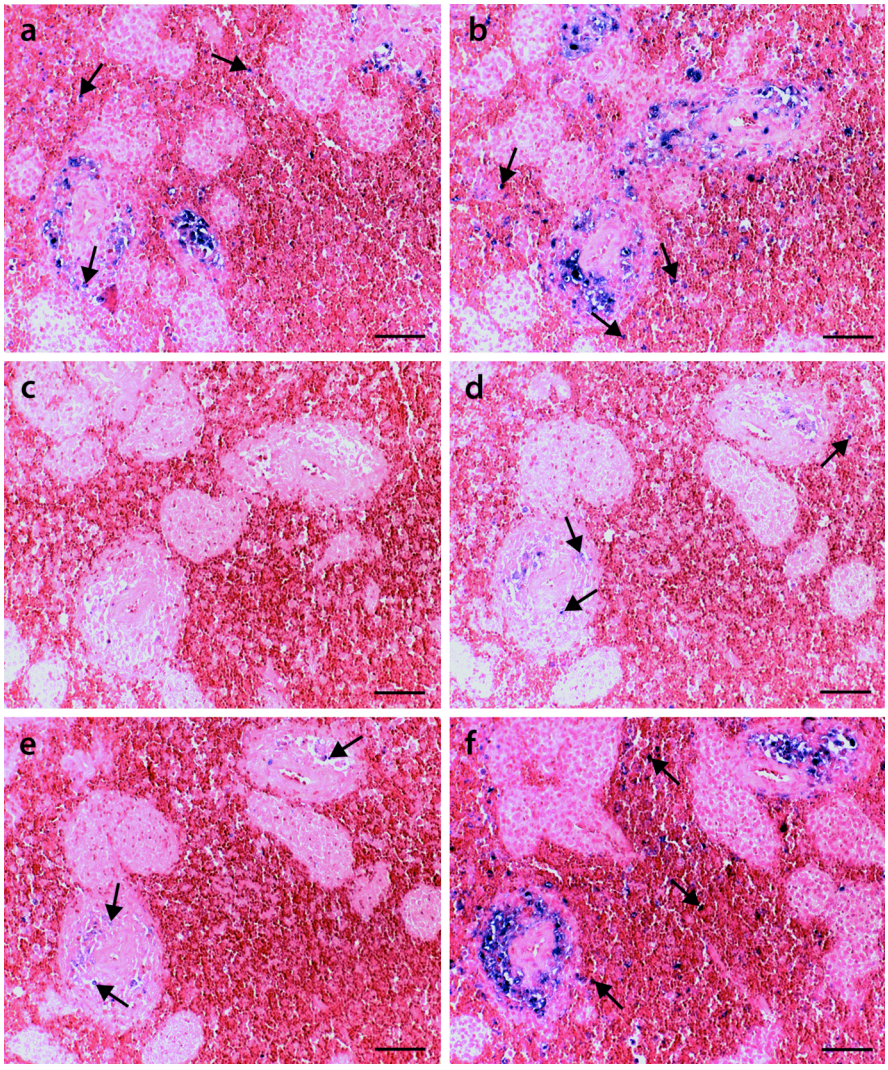

Figure 2. Oligonucleotide hybridization target identification on spleen sections from PMWS diseased piglet tissue. Oligonucleotide ISH on consecutive formalin fixed paraffin embedded spleen sections. These tissue sections were either untreated (a) or, for 1 hour incubated with digestion buffer only (b), for one hour digested with 50 U DNase I (c), for 5 min treated with 50 U DNase I (d), for one hour digested with 20 U DNase I (e), for one hour incubated with an effective Rnase A/Rnase H cocktail (f) followed by ISH with oligonucleotide AB. Arrows indicate PCV2 target presence (blue signals) in periarteriolar lymphatic sheath and probably in histocytes/macrophages distributed throughout the tissue sections. The tissue sections were counterstained with ISH-Red. At the right lower bottom of pictures, bar represents 50 μm.

With less time or lower concentration of DNase I treatment weak signals remained i.e., about 90% of the signal was removed (Figure 2d, 2e). Tissue sections treated with 1 U DNAse I did not reduce signal intensity (data not shown). Reduction of DNA dependent signals was in contrast to tissue sections treated with a combination of RNase A and RNase H (Figure 2f). We did not observe any signal intensity reduction in sections digested with the RNase cocktail over buffer control alone. The combination RNase A and RNase H concentration was sufficient in other experiments to eliminate RNA

dependent signals. Therefore, we concluded that signals we visualized were originating solely from DNA homodimers, namely, oligonucleotide probe and PCV2 DNA. We repeated these DNase and RNase digestions on pig tissue sections with genotype group specific oligonucleotides, A and B, and with reverse, complement Ar and Br oligonucleotides reminiscent to AB oligonucleotide probing, we found that all signals were dependent on the presence of PCV2 DNA (Figure 2).

Cellular co-localization of PCV2a and PCV2b genotype groups

Previously we determined PCV2a and PCV2b genotype groups ratio by PCR amplificate sequencing. ISH hybridization on consecutive tissue sections yielded to same conclusions: pre-epizootic cases showed PCV2a genotype group signal dominance over PCV2b genotype group infections and, epizootic PCV2b genotype group infection governed signals. In figure 3 we show such an example of tonsil tissue from prior to (Figure 3a, 3b) and during epizooty (Figure 3c, 3d).

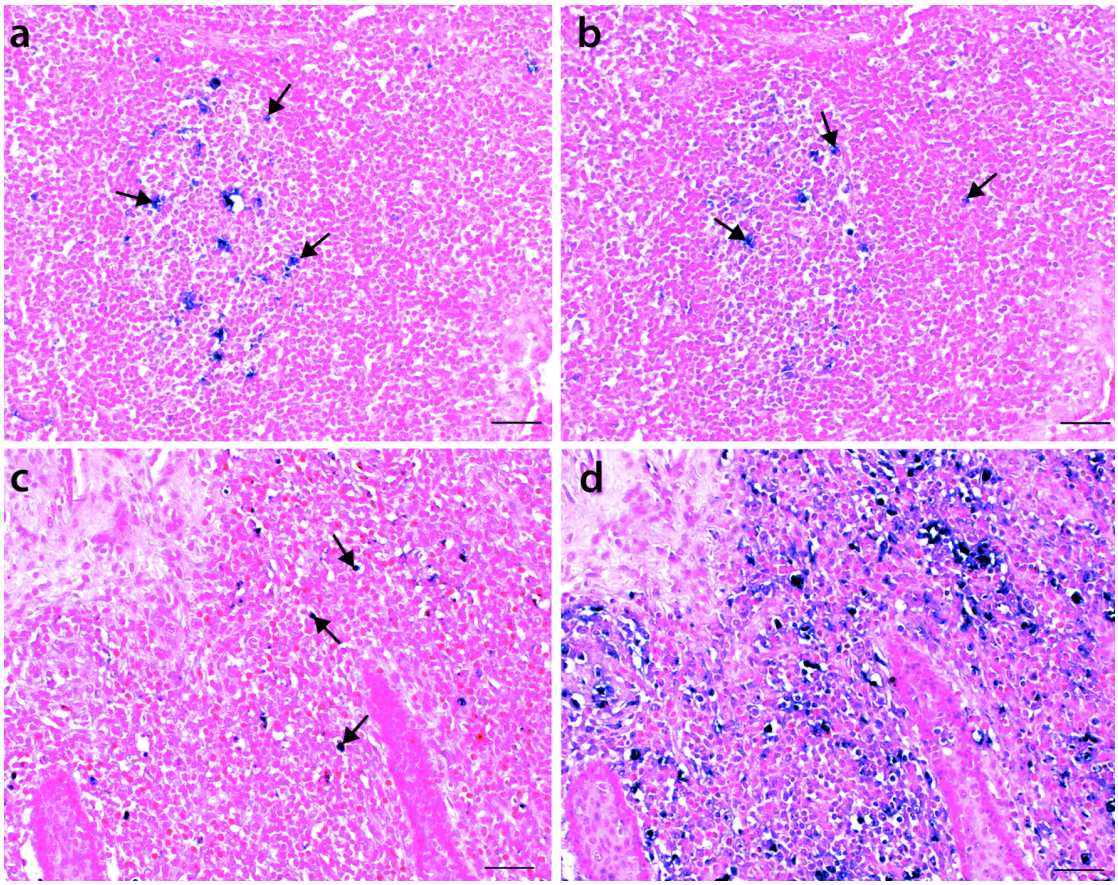

Figure 3. Typical PCV2 genotype group distribution before and during the epizooty. PCV2a/PCV2b genotype groups differential signals of fixed and paraffin embedded tonsil tissue sections prior to (a/b) and during Swiss epizooty (c/d). ISH with oligonucleotide A (a, c) and oligonucleotide B probe (b, d) recognize PCV2a and PCV2b viruses, respectively. Note presence of PCV2 genotype group signals by a blue stain also indicated by arrows. Tissue sections were counterstained with ISH-Red. At the right lower bottom of pictures, bar represents 50 μm.

Additionally, we noticed in these tissue sections and in other experiments that often ISH signals from both genotype groups overlapped with variable genotype group signal intensity. Under the same conditions, none of the oligonucleotides bound unspecifically in any of the negative controls. We questioned whether comparing consecutive tissue sections' signals might perturb results. Hitherto, we established fluorescence in situ hybridization (FISH) to compare genotype group distribution directly on the same tissue section. For FISH we utilized oligonucleotide sequence and hybridization conditions reminiscent to ISH reactions. Specific oligonucleotides were coupled with either Atto 565 for PCV2a genotype group recognition or Dy 630 fluorochrome for PCV2b genotype group identification. The fluorescent colours were heat-stable and clearly distinguishable in laser confocal microscopy. In contrast, the combination Atto 594 and Atto 565 fluorochrom interfered depending on PCV2 DNA content in sections. Additionally, we could not use green channels for laser confocal microscopy as we found insurmountable interference with tissue section's auto-fluorescence. Finally, we were able to establish a 3 colour staining protocol for confocal microscopy: we utilized DAPI blue fluorescent to visualize nuclei, Atto coupled oligonucleotide B and Dy coupled oligonucleotide A for specific PCV2 genotype group DNA labelling. To expedite analysis we compared sections with the help of tissue micro array (TMA). The genotype specific oligonucleotides were designed over the signature motif in the ORF2. They were designed to discriminate between the two major genotype groups, PCV2a and PCV2b, and recognized all subgenotype specific PCV2 variants. The highest sequence variability was chosen around the middle of oligonucleotide hybridization sequence to achieve highest template specificity. We found in all infected tissue sections by ISH, reminiscent of PCR results, both genotype groups, PCV2a and PCV2b, however FISH signals were more specifically defined in individually infected cells (Figure 4). PCV2 was present mostly in the cell cytoplasm and sometimes intra-nuclear.

Nevertheless, genotype group superinfections were found in variable ratios and few cells were single infected by one genotype group. Often a slight labelling of PCV2b or PCV2a genotype group was found in the background of the other dominant genotype group. Presence of both genotype groups was particularly unexpected in tissue from all six piglets with low PCV2 infections (Table) even though it was supported by PCR analysis. Cells with double infections always appeared equal or stronger in fluorescence intensity in either channel compared to genotype group single cell infections.

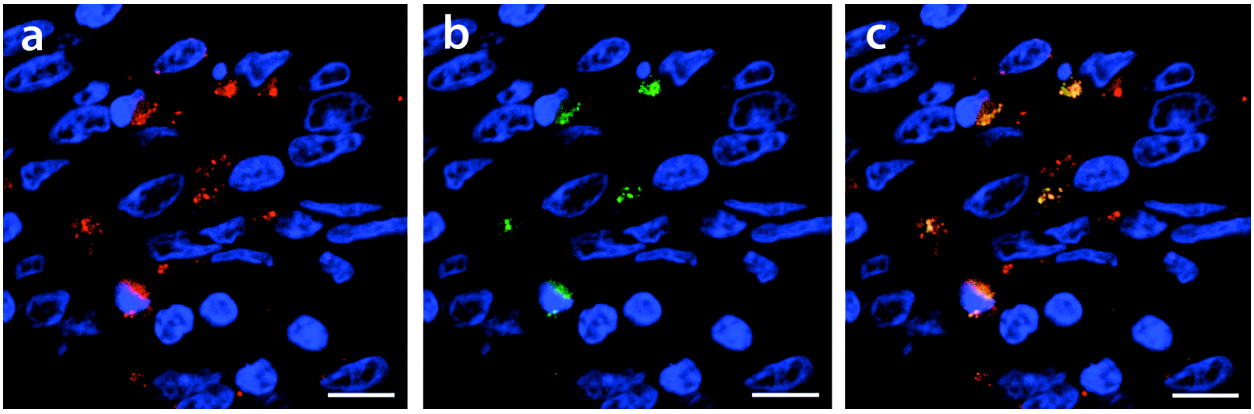

Figure 4. Laser confocal microscopy analysis revealed PCV2a and PCV2b genotype group infections in the same cell. FISH from fixed and paraffin embedded lymph node tissue section of an epizootic PMWS diseased animal. Red signals derived from the PCV2b specific binding oligonucleotide B coupled with Atto chromophore (a). Green represents signals achieved from oligonucleotide A target hybridization labelled with Dy (b). Panel c results from overlay of (a) and (b) pictures. In all panels nuclei are counterstained with DAPI and thus appear blue. The bar indicates 10 μm.

Unexpected intracellular co-replication of the two major PCV2 genotype groups

To identify presence of replicative PCV2 DNA isoform we designed the complementary reverse oligonucleotide to AB oligonucleotide we named ABr oligonucleotide. As ABr oligonucleotide could also potentially recognize RNA derived from ORF2, we verified absence of RNA in tissue sections by three different methods: by reverse transcriptase PCR from RNA virus infected tissue sections (unpublished data), with RNA positive control from Ventana Medical Systems for the detection of mRNA in tissue sections and, DNase/RNase digestions experiments (comparable to method in Figure 2). These data convinced us that ISH and FISH signals are generated dominantly through the presence of target DNA. Signals observed by oligonucleotide ABr hybridization specifically indicated the presence of PCV2 dsDNA. The signals observed were nuclear and cytoplasmic that compared to AB oligonucleotide hybridization signals (Figure 5).

We found in all tissue sections more AB oligonucleotide labelled cells compared to ABr based signals and all cells labelled with ABr oligonucleotide were also strongly stained by AB oligonucleotide specific probe. In cells with both fluorescence markers labelling overlapped almost completely (Figure 5c). Cells with replicative isoform mostly appeared compacted round (Figure 6) compared to single AB oligonucleotide stained cells (Figure 5c).

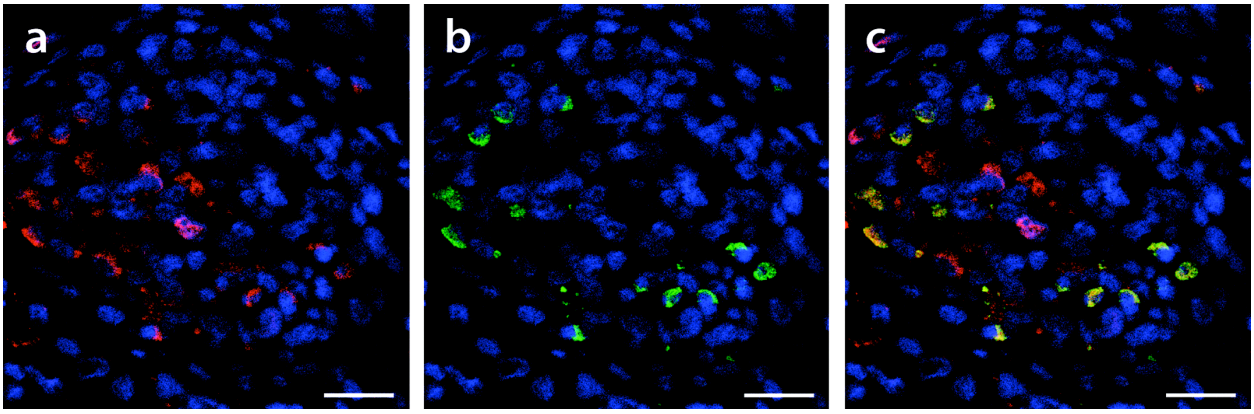

Figure 5. Detection of PCV2 replication by laser confocal microscopy. FISH used specifically for identification of PCV2 dsDNA in PCV2 infected formalin fixed and paraffin embedded spleen tissue. Red signals derived from Atto coupled oligonucleotide AB hybridization identify all PCV2 infection (a). Green labelling was derived from oligonucleotide ABr coupled Dy hybridization (b). Panel (c) is an overlay of pictures (a) and (b). Nuclei are counterstained with DAPI and thus appear blue. The bar indicates 20 μm.

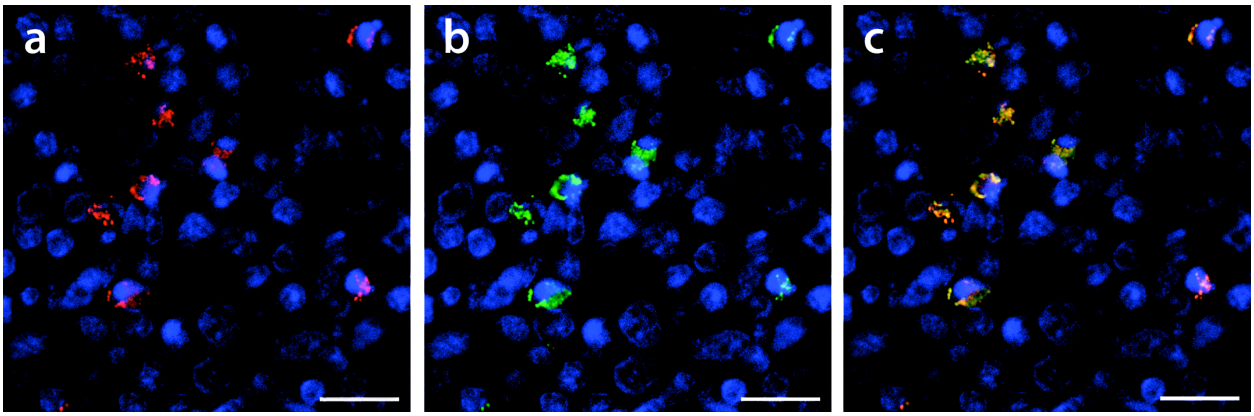

Figure 6. PCV2 genotype groups co-localization and co-replication visualized by a confocal micrograph. Genotype groups superfection and co-replication visualized by FISH of formalin fixed and paraffin embedded tonsil tissue section. Red labelling achieved by hybridization of PCV2a dsDNA specific Atto coupled oligonucleotide Ar (a). Dy coupled oligonucleotide Br target hybridization specific for PCV2b dsDNA appears green in the panel (b). Panel (c) is the result of pictures (a) and (b) overlay. In all panels nuclei are counterstained with DAPI and thus appear blue. The bar indicates 15 μm.

We estimated the ratio dependent on tissue from PCV2 general infection to ds PCV2 DNA replicative isoform, from 1 to 1 to about 50 to 1, respectively. In low infected tissues we hardly detected any PCV2 replicative isoform specific signals. The PCV2 replicative isoform was readily detected in sections with moderate to very high viral antigen loads. Additionally, we observed this isoform in lymphoid organs, such as tonsil, peyer's patches of ileum, lymph nodes, and spleen and to a lesser extent in lung and liver.

As we found many infected cells carrying both major genotype groups albeit with different ratio, it was of particular interest to understand whether these genotypes might co-replicate intracellularly. Hence, we designed a FISH experiment with reverse complement sequence to A and B oligonucleotides, namely with oligonucleotide Ar and

Br, which specifically would recognize genotype group specific replicative isoform. In ISH experiments we confirmed absence of RNA based signals and on TMA sections we verified specificity of genotype group dsDNA recognizing oligonucleotides. Labelling was observed in moderate to high antigen containing tissue sections. Notably, we found in all PCV2 infected tissue sections Ar and Br oligonucleotide generated signal co-localized in the same cell. We observed only minor signal intensity differences, if any, between the specific PCV2 genotype groups cell internal replication (Figure 6c). The ratio between the labellings rarely varied in contrast to genotype specific A and B oligonucleotide labelling. This pattern was observed in many different tissue sections including tonsil, lymph node, spleen, lung and liver.

DISCUSSION

In this study, we demonstrate double genotype group infections in all investigated PCV2 infected pigs and organs. Furthermore, we found *in vivo* cell superinfections and co-replication of the two major PCV2 genotype groups, PCV2a and PCV2b. It explains many recent reports and findings about PCV2 nature in general and in particular the heightened recombination efficiency of these two major PCV2 genotype groups. To our knowledge this is the first report directly showing superinfections of two so closely related virus groups and suggest genotype groups' cooperation for efficient co-replication *in vivo*. These findings give a new twist to syndrome etiology understanding. A major thinking leap is required to connect PCV2 with PMWS and thus cofactors are assumed to be essential to trigger disease. In the new model no cofactors are required to initiate PMWS. Instead we suggest presence of both major genotype groups is essential in addition to host driven autogenic selection of PCV2 genotype group members for disease.

We report 100 % of investigated PCV2 infected pigs carry both genotype groups, PCV2a and PCV2b. These surprising results led to further confirmation by unrelated methods including genotype specific PCR, ISH and FISH. Even IHC negative tissue samples with low virus concentration content were found infected with both genotype groups. Although other research had already reported double infections in pigs, the prevalence was no higher than 25 % of all infected animals [30]. In retrospect, we found double infections in tissue samples of infected animals dating back to 1981. These data support the surprising suggestion that PCV2a and PCV2b co-evolutionary were transferred from birds to pigs [10].

Our concerns needed further evaluation about specificity and sensitivity of the applied methods. To check genotype group specific PCR amplification sensitivity and specificity, we cloned PCV2a and PCV2b sequences. These plasmid constructs were diluted and amplified by corresponding genotype group specific PCR to serve as control. The sensitivity limits were about 10-100 templates per reaction comparable to an efficient amplification. We also investigated cross-reactivity of genotype group specific PCRs: PCR specific for PCV2a amplification was used to amplify PCV2b sequences and vice versa. From both genotype groups specific PCRs, even at the highest concentration in extreme virus infected tissue of 10^7-10^8 templates, no unspecific amplificate was visible. Previously, we identified 42% of all IHC negative cases by PCR as PCV2 infected [19].

We randomly chose six and all of them were positive in ISH and FISH. In these tissues, a few cells were labelled positive for PCV2 DNA in ISH. The signals were confined to centro-follicular cells and immigrated phagocytes, both cell types generally known to contain virus antigen when infected with PCV2. Additionally, we found genotype group specific signals reminiscent to earlier PCR data [19]. These earlier data were based on a PCR reaction that did not discriminate among genotype groups, however by sequencing of amplificate we could define genotype. Newly supported by genotypic PCR reaction, genotypic ISH and FISH, we again observed pre-epizootic PCV2 infected pig tissues were preferentially labelled with PCV2a genotype group specific probe and, during epizooty the ratio reversed to more PCV2b group specific signals. ISH was further validated by screening of PCV2 negative tissue from cat, dog and also PCV2-PCR negative pig tissue. Data indicate that PCV2b genotype group was always present and had not newly occurred even though its occurrence dominates today's epizooty in Switzerland as well as in other countries including USA and Canada. Even as this genetical shift was observed, every PCV2 infected animal and infected organ was found to carry both PCV2a and PCV2b genotype groups. In fact by PCR amplification and sequencing, subgenotypes were additionally identified in the background of genotype groups [19,29].

We demonstrated the presence of both genotype groups in individual cells. Double genotype group infections per cell were not seen sporadically, on the contrary, they were found even in tissue with very few infected cells. Indeed, double PCV2 genotype group infections were more prominent in these tissues. Single cell infections were only observed in moderate to high PCV2 infected tissues. These observations were counterintuitive to expectations, as with low infections we expected more single genotype infected cells; alternatively these PCV2 carrying cells were immigrating phagocytes. In any biological system, one or the other phylogenetically closely related virus has at random, a slight replication advantage over the other, and consequently one genotype would become diluted and completely disappears unless there is a special requirement for the presence of both genotypes. The general assumptions are that closely related viruses are mostly single infections and, their replication would independently be most efficient with a single genotype. In our observations, both PCV2 genotype groups were always seen together during replication, this suggests that both genotype groups are replicating cooperatively *in vivo.* It needs to be seen whether this replication cooperativity can be studied in *in vitro* experiments.

PCV2 is found as two DNA isoforms: ssDNA and dsDNA, the replicative isoform of the virus. In the absence of interference from RNA mediated signals, the replicative dsDNA isoform was distinguished by oligonucleotide ABr mediated ISH or FISH from total PCV2 DNA. To exclude any PCV2 RNA signal perturbation of interpretation, three different approaches by RT-PCR, a general mRNA detection assay from Ventana Medical Systems and, differential DNase/RNase digestions were applied convincing us that observed signals resulted from DNA-DNA interactions. The ISH and FISH observed signals were PCV2 DNA specific. Interestingly, PCV2 replicative isoform was observed in secondary lymphoid organs, including tonsil, peyer's patches of ileum, lymph nodes, and spleen. Additionally, it was also present in lung and liver.

Although we found sporadic single cell infections by PCV2a or PCV2b genotype groups, we also observed more cells infected with both genotype group members with varying infection ratios. This pattern was visible not only in the secondary lymph organs, but also in lung and liver tissue. Efficient replication was observed only in cells containing almost equal amounts of the two genotype group members. We were unable to find any PCV2 infected cell hosting virus replication with one genotype present. Affected cells showed round phenotype, which might be an indication of cellular stress. In neonates, only double genotype infections caused severe disease [34]. This supports our *in vivo* findings that effective pathogenicity needs both genotype groups. Some characteristics of PCV2 can also be seen in geminiviruses, where, phylogentically closely related viruses interspecies recombination correlated with severe disease outbreak [3]**.** However, not all phylogentically closely related viruses allow superinfections [35]. The relaxed replication regulation requirements were not previously observed for DNA viruses in general and in particular with closely related viruses. This is truly a new characteristic for PCV2 and needs to be addressed for other members of the *Circoviridae* family.

We identified PCV2a and PCV2b genotype group co-replication. It is easily imagined that beside these two major genotype groups, subgenotypes may also be found co-replicating in the same manner as subgenotypes recombination of either PCV2a or PCV2b group members [28,36]. This makes sense in the light of virus replication: the virus ssDNA nature and, its propagation by a melting pot rolling cycle mechanism [7]. Every new virus appears first as a ssDNA template, a replication system known to be exceptionally prone to point mutations. The recent accumulation of reports about PCV2 recombination supports the presence of active replicating genotypes and subgenotypes

in the cell. Our findings provide a complete new perspective for a rapid changing genetic PCV2 pool in infected pigs and cells and with it, the occurrence of different virulent PCV2 mutants. We suggest the combination of all these factors and particularly the relaxed replication regulation requirement of PCV2 allows the virus to effectively adapt and obviate the host defence system pressure. Additionally, this heightened mutation rate [10] gives the virus a real angle to zoonosis as in less than hundred years it transferred from birds, to the wild boar and into the domestic pig population.

Most PCV2 disease research focused on additional cofactors, which would be needed to trigger PMWS disease. However, none of these identified cofactors were of importance at the commencement of the Swiss PMWS epizooty [19]. Therefore, we favour a model where a diverse genetic PCV2 pool constantly evolving in host herds, in individual animals and cells explain disease etiology. This would further explain the population dynamic of germination centers for PMWS spread and outbreaks in Switzerland and Great Britain as if a new infectious agent with PCV2 co-emerged [37]. Under this assumption, the creeping nature of the disease in individual animal during maturation involves harmful virus mutation and recombination that leads to increased lymphopenie that finally triggers disease. Additional cofactors accelerate disease course in this model. We will, in further animal infection experiments test this hypothesis.

MATERIALS AND METHODS

Ethics Statement

The tissue samples were taken from routine necropsy operations by the Institute of Veterinary Pathology, University of Zurich, and adhered to standards and regulations of ISO / IEC 17025 (Number STS 255). This study was carried out also in strict accordance with the recommendations of the Swiss Federal Veterinary Office (FVO) and according to the Ethical Principles and Guidelines for Experiments on Animals as formulated jointly by the Swiss Academy of Medical Sciences (SAMS) and the Swiss Academy of Sciences (SCNAT).

Samples and Plasmids

Paraffin-embedded archived necropsy tissues from piglets of the years 1981 through 2009 were selected randomly and arranged in different tissue micro array (TMA) blocks. Mostly, secondary lymphoid tissues including spleen, tonsils, lymph nodes and peyer's patches of the ileum were used. The tissues were of piglets from 2 to 4 months of age. We selected and investigated 42 piglet cases and 6 subclinical immunohistochemistry (IHC) negative cases. PMWS cases are defined by IHC [38] as containing medium to high PCV2 antigen and showing histological lesions and clinical disease symptoms. As negative controls, PCV2 DNA negative secondary lymphoid tissues from piglets and lymphoid tissues from other species such as dogs, a cat and a snake were included. As previously described, the presence of PCV2 antigen was determined by IHC [19]. DNA was extracted from the same samples using the Qiagen tissue kit (Hilden, Germany) according to the manufacturer's instructions.

Plasmids for PCV2 and genotype specific PCR controls were constructed by ligation of PCV2a (AF109398 or AY754018) or PCV2b (DQ923523) PCR fragments (137 bp excluding oligonucleotide sequences) [19] encoding the signature motif into pCR®2.1-TOPO® vector (Invitrogen™). Correct inserts were confirmed with *Eco* RI restriction endnuclease (New England Biolabs© Inc.) digestion and sequencing of the constructs.

DNA preparation and genotype group specific PCR

Total DNA was extracted from 6 μm thin slices of fixed and paraffin embedded piglet tissues using the Qiagen tissue kit (Hilden, Germany) according to manufacturer's instructions. PCR amplification of dominant PCV2 sequence from tissue section was previously described [19]. PCR amplification of PCV2a genotype group specific sequences were achieved using the forward and reverse primers, PCV2F 5' CGY TGG AGA AGG AAA AAY GGC 3' and PCV2R-A 5' GTA GTA TTC AAA GGG TAY AGA GAT 3'. For PCR amplification of PCV2b genotype group specific sequences we utilized PCV2F oligonucleotide and a PCV2b-specific primer PCV2R-B 5' GTA TTC AAA GGG CAC AGA GMG G 3'. We used PCV2a or PCV2b primer set for genotype group specific sequence amplification, for semi-quantitative analysis and sequencing in following reaction mix: A total PCR reaction volume of 50 μl was used with each oligonucleotide at a 1 μM concentration, 1x PCR buffer + $MgCl_2$ (Roche, Molecular Biochemicals), 2 mM $MgCl_2$, 80 μM dNTP (Roche, Molecular Biochemicals), 1.25 U FastStart Taq polymerase (Roche, Molecular Biochemicals) and 1 μl of DNA template. PCR reactions were performed in the TGradient (Biometra®) with 3 min activation of FastStart Taq DNA polymerase at 95 °C, followed by 30 s denaturing at 95 °C, annealing at 54 °C for 60 s, and 60 s elongation at 72 °C for 35 cycles, PCR reaction cycles were completed by 10 min extension reaction at 72 °C. The PCR products were separated on a 1.5% agarose gel by electrophoresis and visualised with UV light after staining with ethidium bromide (Fluka, Sigma-Aldrich) or GelRed™ (Chemie Brunschwig AG, Basel).

Oligonucleotides used for In Situ Hybridization (ISH) and Fluorescence In Situ Hybridization (FISH) analysis

Oligonucleotides were on both ends, 5' and 3', either biotinylated or fluorescent labelled with Atto 565 or dyomics 630 (Dy 630) (Microsynth AG, Switzerland) depending on usage. All oligonucleotide probes were designed to recognize sequences of PCV2 ORF2 excluding PCV1 sequence recognition. AB 5' CCATCTTGGCCAGATCCTCCGCCG 3' and ABr oligonucleotide 5' CGGCGGAGGATCTGGCCAAGATGG 3' are both 24mers. The sequence of ABr is exactly reverse, complement to AB oligonucleotide sequence. Both oligonucleotides will recognize all described PCV2a, PCV2b and PCV2c genotype group members. They differ in that AB oligonucleotide (AB sequence identical with sense sequence) -

recognizes, in the absence of RNA, single strand (ss) and double strand (ds) PCV2 specific DNA and ABr recognizes exclusively PCV2 dsDNA isoform. And oligonucleotide A 5' GGGGACCAACAAAATCTCTRTACC 3' and B 5' GGCTCAAACCCCCKCTCTGTGC 3' distinguish between PCV2a and PCV2b genotype groups, respectively. Since B oligonucleotide is more GC rich, A oligonucleotide is 2 bp longer to parallel hybridization conditions. Both genotype specific oligonucleotides were designed over the PCV2 ORF2 signature motif, thus the critical nucleotides are located mostly towards the middle of hybridization sequence [39]. Oligonucleotides Ar 5' GGTAYAGAGATTTTGTTGGTCCCC 3' and Br 5' GCACAGAGMGGGGGTTTGAGCC 3' were designed to distinguish PCV2a and PCV2b genotype groups in the PCV2 ORF2. Additionally, both are of the complement and reverse sequence of A and B oligonucleotides, hence, in the absence of RNA target they would recognize solely the dsDNA isoform of the virus.

ISH with 5' and 3' biotinylated DNA oligonucleotide probes

Before ISH commencement we manually deparaffinized mounted tissue sections. We used DISCOVERY™ instrument (Ventana Medical Systems) to establish PCV2 specific ISHs'. Experimental protocols were run with help of DISCOVERY™ software. We standardized pre-treatment on formalin-fixed, paraffin-embedded tissue sections by using following RIBOMAP™ reagents: we treated slices 28 min with RIBOPREP™ and 8 min with RIBOCLEAR™ at RT before Protease I (Ventana Medical Systems) incubation for 4 min at 37 °C. Next, the tissue sections were overlaid with corresponding 5' and 3' biotinylated oligonucleotides (Microsynth AG, Switzerland) and hybridization solution consisting of 6x SSC, 5x Denhardt solution, 12% dextran sulphate sodium salt and distilled water. The probes were diluted to a final concentration between 2-10 pmol in hybridization solution dependent on negative controls and signal intensity. Afterward, overlaid slides were subjected to a 4 min denaturing step at 90ºC followed by hybridization for two hours at 37 °C. Slides with mounted tissue were washed in two stringency washes of 2x RIBOWASH™ each at 47 ºC for four minutes. Next, samples were treated with RIBOFIX™ reagent for 24 min to fix stably remaining oligonucleotide probe to target DNA. A washing step automatically followed with 1x reaction buffer (Ventana Medical Systems). For signal detection, tissue sections were incubated for 1 hour with BLUEMAP™ detection kit (Ventana Medical Systems) and Pab-Block (Ventana Medical Systems) that reduced unnecessary background staining. Slides were counterstained using ISH-Red (Ventana Medical Systems) for 20 minutes. Post-

counterstain slides were washed shortly with soap and distilled water, dehydrated and mounted.

FISH with 5' and 3' fluorochrome Atto 565 or Dy 630 labelled oligonucleotide probes for laser confocal microscopy

Mycrosynth AG (Switzerland) synthesized 5' and 3' chromophores coupled oligonucleotides with either Atto 565 or Dy 630.

ISH protocol on the DISCOVERY™ instrument (Ventana Medical Systems) provided groundwork for FISH experimental steps: both protocol procedures were identical to step with post-fixation with RIBOFIX™ reagent for 24 minutes. Separately for FISH, we washed slides twice with distilled water followed by a nuclear staining with 1 µg/ml diamidino-2-phenylindole (DAPI) (AppliChem GmbH, Germany) in methanol for 20 min. After slides were washed again in distilled water and air-dried prior to application of aqueous mounting medium, Immu-Mount (Thermo™, Pitsburgh) and cover slip.

For detection of signals we used a Leica SP5 laser confocal scanning microscope with three lasers and with four confocal fluorescence detectors at the Center for Microscopy and Image Analysis (University of Zurich, Switzerland). We used the diode laser excitation at 405 nm for DAPI signal visualization and the Helium Neon laser excitations at 561 nm or at 633 nm for Atto 565 or Dy 630 detection, respectively.

Confocal pictures were analysed with Imaris 6.3.0 (Bitplane, Scientific Software) a multicolour and 3 D /4 D image processing software.

Mounted tissue sections digested either with DNase or RNase enzyme

ISH protocol on the DISCOVERY™ instrument (Ventana Medical Systems) was adjusted for this procedure and slide mounted tissue sections were subjected to either a desoxyribonuclease I (DNase I) or ribonuclease (RNase) digestion before oligonucleotide hybridization. Consecutive tissue section slides were preferentially used. In brief, tissue sections were for DNA or RNA digestion overlaid with 300 µl enzyme aqueous buffer solution and incubated at 37 °C in a humidifying chamber for the times indicated. We overlaid concentration of 1-100 U DNase I (Fermentas) per tissue section, which were incubated for 1 hour at 37 °C. Other tissue sections were incubated with RNase cocktail for 1 hour at 37 °C. We used a combination of 83 µg/ml RNase A (Fermentas) and 83 U/ml endoribonuclease RNase H (Biolabs) to digest tissue sections

with possible target RNA presence. These enzyme concentrations were chosen at the higher end of concentration recommendations of manufacturer.

After tissue slices were washed twice with reaction buffer (Ventana Medical Systems) and introduced into DISCOVERY™ instrument (Ventana Medical Systems) we continued the experiment immediately with the step of oligonucleotide hybridization.

REFERENCES

1. Gibbs MJ, Weiller GF (1999) Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc Natl Acad Sci U S A 96: 8022-8027.
2. Katoh H, Ogawa H, Ohya K, Fukushi H (2010) A Review of DNA Viral Infections in Psittacine Birds. J Vet Med Sci
3. Zhou X, Liu Y, Calvert L, Munoz C, Otim-Nape GW et al. (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J Gen Virol 78: 2101-2111.
4. Allan GM, Ellis JA (2000) Porcine circoviruses: a review. J Vet Diagn Invest 12: 3-14.
5. Meehan BM, Creelan JL, McNulty MS, Todd D (1997) Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol 78: 221-227.
6. Steinfeldt T, Finsterbusch T, Mankertz A (2006) Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep' proteins of porcine circovirus type 1. J Virol 80: 6225-6234.
7. Faurez F, Dory D, Grasland B, Jestin A (2009) Replication of porcine circoviruses. Virol J 6: 60.
8. Liu J, Chen I, Kwang J (2005) Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J Virol 79: 8262-8274.
9. Mankertz A, Caliskan R, Hattermann K, Hillenbrand B, Kurzendoerfer P et al. (2004) Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Vet Microbiol 98: 81-88.
10. Firth C, Charleston MA, Duffy S, Shapiro B, Holmes EC (2009) Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. J Virol 83: 12813-12821.
11. Kappe EC, Halami MY, Schade B, Alex M, Hoffmann D et al. (2010) Bone marrow depletion with haemorrhagic diathesis in calves in Germany: characterization of the disease and preliminary investigations on its aetiology. Berl Munch Tierarztl Wochenschr 123: 31-41.
12. Tischer I, Bode L, Apodaca J, Timm H, Peters D et al. (1995) Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch Virol 140: 1427-1439.
13. Bernstein CN, Nayar G, Hamel A, Blanchard JF (2003) Study of animal-borne infections in the mucosas of patients with inflammatory bowel disease and population-based controls. J Clin Microbiol 41: 4986-4990.
14. Lorincz M, Csagola A, Biksi I, Szeredi L, Dan A et al. (2010) Detection of porcine circovirus in rodents - Short communication. Acta Vet Hung 58: 265-268.
15. Ellis J, Hassard L, Clark E, Harding J, Allan G et al. (1998) Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J 39: 44-51.
16. Segales J, Allan GM, Domingo M (2005) Porcine circovirus diseases. Anim Health Res Rev 6: 119-142.

17. Segales J, Domingo M (2002) Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet Q 24: 109-124.
18. Madec F, Rose N, Grasland B, Cariolet R, Jestin A (2008) Post-weaning multisystemic wasting syndrome and other PCV2-related problems in pigs: a 12-year experience. Transbound Emerg Dis 55: 273-283.
19. Wiederkehr DD, Sydler T, Buergi E, Haessig M, Zimmermann D et al. (2009) A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet Microbiol 136: 27-35.
20. Segales J, Olvera A, Grau-Roma L, Charreyre C, Nauwynck H et al. (2008) PCV-2 genotype definition and nomenclature. Vet Rec 162(26): 867-868.
21. Cheung AK, Lager KM, Kohutyuk OI, Vincent AL, Henry SC et al. (2007) Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch Virol 152: 1035-1044.
22. Dupont K, Nielsen EO, Baekbo P, Larsen LE (2008) Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet Microbiol 128: 56-64.
23. Carman S, McEwen B, DeLay J, van Dreumel T, Lusis P et al. (2006) Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Can Vet J 47: 761-762.
24. Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ (2004) Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J Virol 78: 13440-13446.
25. Hasslung F, Wallgren P, Ladekjaer-Hansen AS, Botner A, Nielsen J et al. (2005) Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Vet Microbiol 106: 49-60.
26. Opriessnig T, McKeown NE, Zhou EM, Meng XJ, Halbur PG (2006) Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. J Gen Virol 87: 2923-2932.
27. Allan GM, McNeilly F, McMenamy M, McNair I, Krakowka SG et al. (2007) Temporal distribution of porcine circovirus 2 genogroups recovered from postweaning multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern Ireland. J Vet Diagn Invest 19: 668-673.
28. Olvera A, Cortey M, Segales J (2007) Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357: 175-185.
29. Grau-Roma L, Crisci E, Sibila M, Lopez-Soria S, Nofrarias M et al. (2008) A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol 128: 23-35.
30. Hesse R, Kerrigan M, Rowland RR (2008) Evidence for recombination between PCV2a and PCV2b in the field. Virus Res 132: 201-207.
31. Kim HK, Luo Y, Moon HJ, Park SJ, Keum HO et al. (2009) Phylogenetic and recombination analysis of genomic sequences of PCV2 isolated in Korea. Virus Genes 39: 352-358.
32. Ma CM, Hon CC, Lam TY, Li VY, Wong CK et al. (2007) Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. J Gen Virol 88: 1733-1737.
33. Cheung AK (2007) Homologous recombination plays minor role in excision of unit-length viral genomes from head-to-tail direct tandem repeats of porcine circovirus during DNA replication in Escherichia coli. Arch Virol 152: 1531-1539.
34. Harding JC, Ellis JA, McIntosh KA, Krakowka S (2010) Dual heterologous porcine circovirus genogroup 2a/2b infection induces severe disease in germ-free pigs. Vet Microbiol

35. Ramirez S, Perez-del-Pulgar S, Carrion JA, Coto-Llerena M, Mensa L et al. (2010) Hepatitis C virus superinfection of liver grafts: a detailed analysis of early exclusion of non-dominant virus strains. J Gen Virol 91: 1183-1188.
36. Lefebvre DJ, Van Doorsselaere J, Delputte PL, Nauwynck HJ (2009) Recombination of two porcine circovirus type 2 strains. Arch Virol 154: 875-879.
37. Woodbine KA, Medley GF, Slevin J, Kilbride AL, Novell EJ et al. (2007) Spatiotemporal patterns and risks of herd breakdowns in pigs with postweaning multisystemic wasting syndrome. Vet Rec 160: 751-762.
38. McNeilly F, McNair I, Mackie DP, Meehan BM, Kennedy S et al. (2001) Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch Virol 146: 909-922.
39. Naiser T, Ehler O, Kayser J, Mai T, Michel W et al. (2008) Impact of point-mutations on the hybridization affinity of surface-bound DNA/DNA and RNA/DNA oligonucleotide-duplexes: comparison of single base mismatches and base bulges. BMC Biotechnol 8: 48.

ACKNOWLEDGEMENTS

We are indebted to Drs. Urs Ziegler and Andreas Kaech and the research team at the Center for Microscopy
and Image Analysis (University of Zurich) for all their help. We would like to thank Roseline Weilenmann at the Institute of Veterinary Pathology for her dedicated technical support and Jeanne Peter at the Graphic Department for her professional assistance.

Curriculum Vitae

Name, First name	Khaiseb Siegfried
Date of birth	10.02.1976
Place of birth	Outjo
Nationality	Namibian
Home City	Windhoek
	Educations:
1982 – 1998	ST. Micheal Primary School, ST. Michael, Namibia
1990 – 1994	Paresis Secondary High School, Otjiwarongo, Namibia • Senior Certificate, Standard 10 • Certificate, Highest marks in Agricultural Science (09.10.1992) • Certificate, Best Learner in Standard 9 (20.10.1993) • Merit Award – Renovation Project at School (20.10.1993) • Certificate, Highest marks in Mathematics Standard 10 (14.10.1994)
1995 - 1997	Neudamm Agricultural College, Windhoek, Namibia • Three Year National Diploma in Agriculture, with the Third-Year Specialization in Livestock Production & Commercial Business Management
	Studies:
1999 – 2005	Kazan State Academy of Veterinary Medicine, Kazan, Russia
30.06.2005	• Diploma: Doctor of Veterinary Medicine
	Dissertation:
2008 - 2010	Writing of the dissertation under scientific supervision by Dr. Enrico Brugnera on the Institute of Veterinary Pathology, Vetsuisse Faculty of Zurich, Switzerland, Director Head of Institution: Prof. Dr. Andreas Pospischil
	Employments:
2006 -	Ministry of Agriculture Water and Forestry, Windhoek, Namibia as State Veterinarian Windhoek (Khomas region)